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Natural variants of human adenovirus type 3 provide evidence for relative genome stability across time and geographic space

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ABSTRACT

Human adenovirus type 3 (HAdV-B3) has an apparently stable genome yet remains a major circulating and problematic respiratory pathogen. Comparisons of the prototype genome to genomes from three current field strains, including two isolated from epidemics, and a laboratory strain, yielded small-scale nucleotide variations across 50 years of time and space (U.S. and China). This is in contrast to the recombination events that have been reported recently for HAdV genomes. Recombinant genomes have been identified in emergent HAdV pathogens and is a pathway for the molecular evolution of types. These two contrasting views of HAdV genome stability have repercussions in the development and use of vaccines for countering HAdV-B3, as well as in the continued effectiveness of vaccines developed against earlier and current circulating types of HAdV.

Introduction

Human adenovirus (HAdV) is a double-stranded DNA virus that was among the first respiratory viruses characterized (Hilleman and Werner, 1954; Rowe et al., 1953). They are implicated in a wide range of human diseases, including respiratory, ocular, metabolic, renal and gastrointestinal; and they impact immunocompromised individuals as well as young, healthy populations (Echavarria, 2008). The natural variation, over a time period of 50 years and across geographic space (U.S. and China) of HAdV-B3 at the whole genome level is reported, comparing the prototype GB strain, which was isolated from a "common cold volunteer" (USA, 1953), with a field strain recovered from a patient in the U.S. nearly 50 years later, and compares both with two recent South China epidemic strains (Zhang et al., 2006), as well as with a laboratory-circulating GB strain from Europe (Sirena et al., 2005). The findings indicate a remarkable conservation and stability of sequences within the genomes across the 50-year time span and across geographic distances. These data stand in contrast to the recombination-driven new isolates that are being characterized recently using genomic and computational analyses (Walsh et al., 2009; Yang et al., 2009).

HAdV-B3, a member of the B1 subspecies, causes acute respiratory disease (ARD). This pathogen is identified in epidemics, is highly virulent and is associated with clinical manifestations of considerable severity including residual lung damage and fatal outcomes (Ryan et al., 2002). HAdV-B3 is found in outbreaks in the U.S., for example, reported in children and military recruits (Ryan et al., 2002), and is a global concern, for example, reported in Asia (Kim et al., 2003; Li et al., 1996) and South America (Kajon et al., 1996), which echo earlier reports of HAdV-B3 isolates across six continents (Li and Wadell, 1988). Restriction enzyme (RE) pattern analysis from those studies revealed limited genome changes over time and location, with overall genome stability, e.g., HAdV-B3a dominating in Japan from 1962 to 1988, with occasional HAdV-B3a8 and B3c reported in the same population (Shiao et al., 1996); and HAdV-B3a2 dominating in China from 1962 to 1988, with occasional HAdV-B3a4, B3a5 and B3a6 (Li and Wadell, 1988; Li et al., 1996). However, it should be noted that HAdV-B3 is not immune to recombination events, for example, a putative recombination event involving HAdV-B3 has been hypothesized as a mechanism resulting in the emergence of HAdV-B7h, characterized in a South American (south cone) outbreak. This strain is apparently more devastating than other HAdV-B7 strains and contains an HAdV-B3-like fiber (Kajon and Wadell, 1996).

In contrast to this apparent stability of these HAdV-B3 genomes, HAdV in general are known to undergo recombination. Earlier studies demonstrated *in vitro* recombination (Boursnell and Mautner, 1981a) as well as illegitimate recombination involving foreign DNA (Ling,

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Manos, and Gluzman, 1982). These are useful properties for biotechnology (Bernt et al., 2002). The genomes also undergo recombination *in vivo* (Boursnell and Mautner, 1981b; Crawford-Miksza and Schnurr, 1996; Lukashev et al., 2008; Mautner and Mackay, 1984; Williams et al., 1975), leading to discussions of recombination as a driving force in HAdV type evolution (Crawford-Miksza and Schnurr, 1996). Apparent recombinant HAdV isolates, isolated from outbreaks, have been reported in the literature as "intermediates", with virus characterization based on serum neutralization and/or limited molecular typing. These have differing properties when examined at their hexon and fiber determinants. For example, strain 14/11 (hexon/fiber) was reported as a respiratory pathogen (Hierholzer and Pumarola, 1976); and more recently, "intermediates" of species D were reported (Crawford-Miksza and Schnurr, 1996; Engelmann et al., 2006; Ishiko et al., 2008). One of these (Engelmann et al., 2006) has been resolved at the genome level, resulting in the recognition of a new "type", HAdV-D53, containing several recombination events that resulted in an emergent HAdV pathogen that causes highly contagious epidemic keratoconjunctivitis (EKC) (Walsh et al., 2009). Thus, with the application of DNA sequencing technology and whole genome computational analyses, there is now additional "primary sequence" evidence to support the hypothesis that genome recombination drives the molecular evolution of HAdV types (Crawford-Miksza and Schnurr, 1996).

Within the context of the *in vitro* recombination potential of the HAdV genome and with the recent genome characterizations of recombination, i.e., "large-scale" genome changes, it is remarkable that these strains of circulating HAdV-B3 have relatively stable genomes, extending a minimum of 50 years, with these particular strains still remaining major public health problems as respiratory pathogens globally. This unusual genome stability was also noted in earlier studies showing that the HAdV-E4p (isolated in the 1950s) and HAdV-E4vac (vaccine strain used in the 1970s and presumably the circulating field strain) genomes as well as the HAdV-B7p and HAdV-B7vac (similar time line) genomes are remarkably conserved as well, albeit across a minimum of 20 years (Purkayastha et al., 2005c). Together, these three observations provide examples into an alternative, yet successful, mode of molecular stability of HAdV genomes, in contrast to the recombination-driven mechanism. These data serve to reinforce the suitability of prophylaxis by vaccines, and suggest that the vaccines developed against earlier circulating strains are still effective today, after 20 and 50 years for HAdV-E4, B7 and B3 (Lyons et al., 2008; Zhang et al., 2009) and point to the potential effectiveness of HAdV vaccines being redeveloped. Genome stability is also a desired property in the application of these genomes as gene delivery vectors.

Results

Genome sequence comparison

The gross genome parameters of all five HAdV-B3 viruses are remarkably similar for these strains isolated at different times (1953, 1997, 2004 and 2005) and across two continents (Asia and North America). The reference and the circulating laboratory (HAdV-B3 GB2) genomes differ by only two nucleotides (35,345 bp and 35,343 bp); the field strain genomes, NHRC 1276, GZ1 and GZ2, are remarkably similar in length (35,265 bp, 35,273 bp, and 35,269 bp). Shown in Table 1, the reference HAdV-B3 GB strain displays the greatest nucleotide percent identity to the other HAdV-B3 strains (~98%) and other members of the HAdV-B1 species than to members of other HAdV species (Table 1). The percent identity between HAdV-B3 GB and HAdV-11 (B2 species) is lower at 82.7%. There is a dramatic decrease in percent identity between the GB strain and members of species A, B, C, D, E, and F. These values range between 61% and 74%.

Table 1
Genome identities of selected adenoviruses versus HAdV-B3 GB. The GC contents of these adenoviruses are also shown.

Type/species	Genome identity (%)	GC (%)
HAdV-B3 GB	100[a]	51.0
HAdV-B3 NHRC 1276	98.2	50.8
HAdV-B3 GZ1	98.2	51.0
HAdV-B3 GZ2	98.2	51.0
HAdV-B7	96.4	51.0
HAdV-B16	94.3	51.3
HAdV-B21	92.8	51.3
HAdV-B50	92.8	51.2
SAdV-21	86.3	51.2
HAdV-B11	82.7	48.9
HAdV-A12	61.8	46.5
HAdV-C5	64.4	55.2
HAdV-D9	67.4	57.1
HAdV-E4	73.9	57.7
SAdV-25	73.7	58.9
HAdV-F40	61.5	51.2
HAdV-F41	61.3	51.0
TSAdV-1	53.3	50.0

[a] HAdV-B3 GB against itself.

Not surprisingly, the tree shrew adenovirus shows a low 53% identity with HAdV-B3 GB.

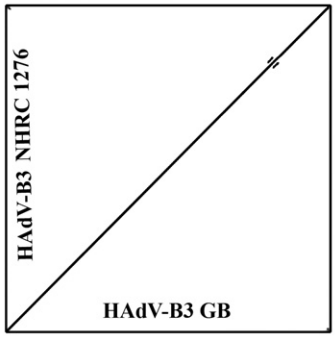
High resolution analysis of these genomes at the nucleotide level reveals remarkable conservation. Fig. 1 presents three views of the whole genome analysis. PipMaker dot plot analysis yields unbroken diagonal lines, indicating high degree of identity across the genomes (Fig. 1A). A small repetitive sequence, within the E3 region, is noted. This repetitive sequence is interesting as it appears in the HAdV-B7 sequences as well, but not in the C and E species sequences to date (Lauer et al., 2004; Purkayastha et al., 2005a,b,c), nor HAdV-D53 (Walsh et al., 2009). The potential significance is unknown, however, the E3 region is involved in host immune response evasion (Kajon et al., 2005).

The numbers of mismatches and gaps in the field strains, with respect to the reference HAdV-B3 GB strain, are also similar, as shown in Fig. 1B. Fig. 1C shows selected deletions and insertions present in the field strains with respect to the reference strain. A 9-bp deletion between nucleotide positions 8449 and 8550 shortens three overlapping proteins by 3 amino acids in the HAdV-B3 NHRC 1276 strain. This corresponds to the dotted box labelled as "1" in Fig. 1C. The affected proteins are the E2B terminal protein precursor, the E2B DNA polymerase and a 12.6 kDa hypothetical protein. An 8 bp insertion occurs in the L1 region of the field strains and does not affect protein coding potential. This corresponds to the hatched box noted as "2" in Fig. 1C. There is a 4 amino acid deletion (QAPS) in the 100 kDa hexon assembly associated protein of the field strains due to a 12 bp deletion in the L4 region, which corresponds to the dotted box labelled as "3" in Fig. 1C. A number of deletions occur in the E3 region of the field strains and these include deletions of 9, 14, 11, and 23 bp. These are shown in the dotted box highlighted as "4" in Fig. 1C. As these deletions occur in a non-coding region of E3, they do not affect the protein coding potential.

Whole genome phylogenetic analysis

Whole genome phylogeny analysis shows these genomes branching in a subclade within the B1 group (Fig. 2). The field strains HAdV-B3 GZ1 and GZ2 are more closely related to each other than they are to HAdV-B3 NHRC 1276. As a validation of this whole genome phylogenetic approach, HAdV-E4 groups together with the other HAdV-E species members. The HAdV-B2 species members 11, 14, 34, and 35 also group together, as well as the species C, D, and F members.

A)



B)

Strain	Mismatches	Gaps
HAdV-B3 NHRC 1276	521	108
HAdV-B3 GZ1	546	104
HAdV-B3 GZ2	545	106

C)

Fig. 1. Whole genome sequence analysis. (A) PipMaker dot blot comparing HAdV-B3p with field strain NHRC 1276 (http://pipmaker.bx.psu.edu/cgi-bin/pipmaker?basic). A short repetitive element is displayed as two short lines on either side of the main diagonal. (B) Mismatches and gaps between the reference HAdV-B3p and the field strains are characterized and displayed. Gaps, defined by PipMaker, are the number of nucleotides inserted or deleted, and mismatches are base substitutions. (C) Selected insertions, based on sizes noted, (hatched boxes) and deletions (dotted boxes) reflect differences in the field strains relative to each other and the reference genome: box 1 is a 9 bp deletion in the E2B region; box 2 notes 8 bp insertions in the L1 region; box 3 shows 12 bp deletions in the L4 region; and box 4 is a collection of closely spaced deletions of 9 bp, 14 bp, 11 bp and 23 bp in the E3 region.

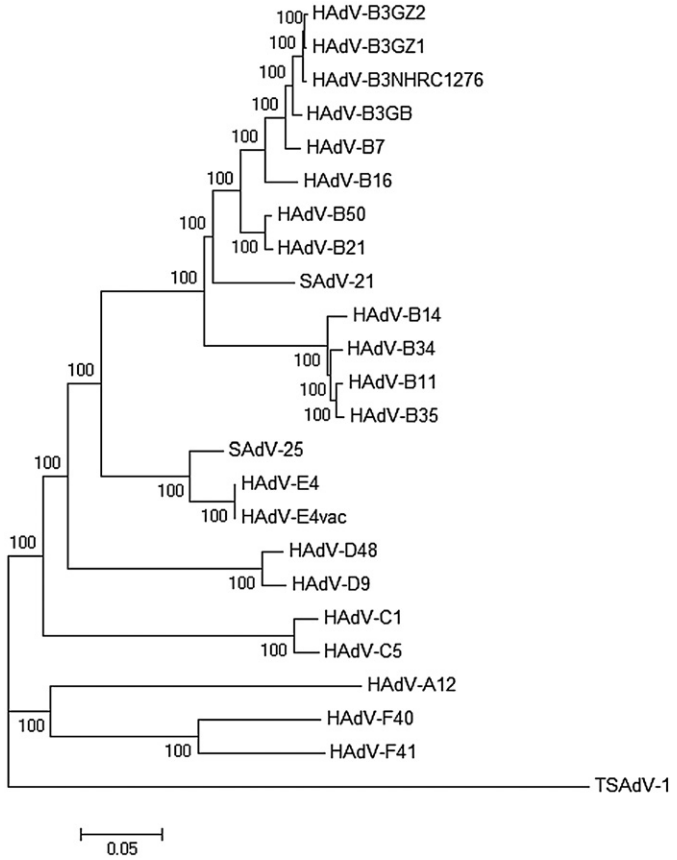


Fig. 2. Whole genome phylogenetic analysis. A neighbor-joining tree of several HAdV whole genome sequences reveals that the HAdV-B3 strains cluster with the other members of the B1 species. The numbers shown represent percentages of 1000 bootstrap replications. The scale bar is in units of nucleotide substitutions per site.

Recombination analysis

Analysis of potential recombination using protein identities, with the GB strain as reference, and software tools, e.g., SimPlot and Bootscan, reveal no clear events (data not shown). This is in contrast to the recent reports of recombination of penton, hexon and other genome sequences characterized using whole genome analysis and apparently driving the molecular evolution of HAdV types and emergence of new pathogens (Robinson et al., 2009; Walsh et al., 2009; Yang et al., 2009).

Restriction enzyme analysis

Whole genome data allow multiple and more accurate *in silico* RE analyses. As a bridge to the important observations and data in the literature, and to synchronize, compare, and make full use of still-relevant wet-bench data in the literature (Li et al., 1996; Shiao et al., 1996), several *in silico* RE digests were performed with these genomes. All three field strains display the HAdV-B3a RE patterns, with one representative RE pattern shown (Fig. 3), reconfirming the genome type of the HAdV-B3 GZ1 and GZ2 strains as identified originally (Zhang et al., 2006). Of the RE analyses performed (*Bam*HI, *Bcl*I, *Bgl*II, *Hpa*I, *Sal*I and *Sma*I), only *Bgl*II and *Sma*I showed slight differences between HAdV-B3 NHRC 1276 and the HAdV-B3 GZ1 and GZ2 genomes, which reflects the whole genome data.

Proteome analysis

The HAdV-B3p and the NHRC 1276 genomes were annotated using methods earlier (Lauer et al., 2004; Purkayastha et al., 2005a, b,c). *In silico* proteome analyses allow an understanding of the implications of the relatively minor changes in the primary nucleotide sequences. Table 2 shows the differences in proteins between the HAdV-B3 GB reference strains and the field strains. In

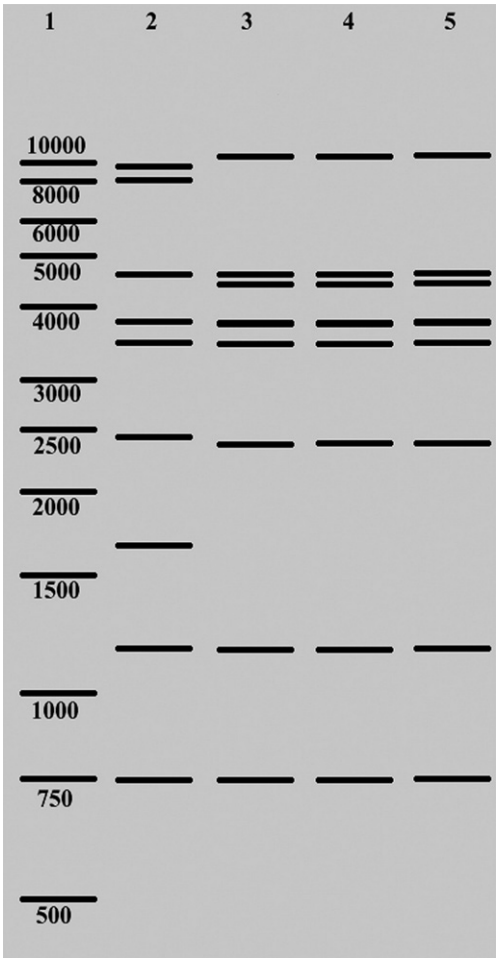


Fig. 3. *In silico* restriction enzyme analysis. To compare the field strains with earlier genome types reported in the literature, a series of RE analyses were performed. A representative BamHI pattern is shown. All three field strains, despite their nucleotide sequence differences, show a common pattern consistent with HAdV-B3a. Lane 1 contains the molecular size markers; lane 2 is HAdV-B3p; lane 3 is NHRC 1276; lane 4 is GZ1; and lane 5 is GZ2.

this table, the "–" symbol means that the protein is not annotated and confirmed to be absent in the annotation due to truncation. The 20.6 -kDa protein is found in both GB genomes, but is present as nonsense mutations in the three field strains. A detailed view of this difference, as an Artemis screenshot, displays the syntenous regions for comparison, Fig. 4. This 20.6 -kDa protein is truncated by a stop codon in the field strains (the SNP was reconfirmed by additional sequencing). In the E2B region, a 19 -kDa protein is annotated for HAdV-B3 GB and HAdV-B3 GB2, but is not found in the field strains. As the other three proteins in this particular region have distinct functions in HAdV DNA replication, this may represent a critical difference; therefore, further wet-bench work is indicated by this bioinformatics result.

Two stop codons truncate this protein in the field strains, shown in Table 2, explaining why it was not annotated originally. Additionally, HAdV-B3 NHRC 1276 has a putative 12.6 kDa hypothetical protein in the E2B region, which is missing in the annotation of the other four strains. A re-inspection of those genomes shows that this protein is truncated by two stop codons in the prototype strains and one stop codon in the GZ1 and GZ2 strains. A E3 9 -kDa glycoprotein, found in both HAdV-B3 GB and GB2, is not annotated in any of the three field strains due to truncation by multiple stop codons. Again, wet-bench research is needed to determine the importance of these computational genomic predictions.

Table 2
In silico proteome differences.

HAdV-B3 GB	HAdV-B3 GB2	HAdV-B3 NHRC 1276	HAdV-B3 GZ1	HAdV-B3 GZ2
20.6 kDa protein	*	–	–	–
19 kDa protein	*	–	–	–
–	–	12.6 kDa hypothetical protein	–	–
E3 9 kDa glycoprotein	*	–	–	–

Presented are the coding sequences that differed from the HAdV-B3p GB strain. HAdV-B3p GB and HAdV-B3a NHRC 1276 genomes are newly annotated (51 and 49 potential coding sequences, respectively). Each genome annotation is reanalyzed to ensure uniformity, that is, if a coding sequence was missing, or present, in one genome uniquely, the syntenous region of other genomes was reanalyzed to account for its actual presence or absence. If annotated uniquely for a genome other than the reference HAdV-B3p GB, then the protein is noted in that column. "*" denotes homolog and "–" denotes confirmed absence. The 20.6 kDa, 19 kDa proteins and 12.6 kDa proteins are in the E2B region.

Discussion

While HAdV genomes are apparently prone to changes via recombination (Robinson et al., 2009; Walsh et al., 2009; Yang et al., 2009), a currently circulating HAdV-B3 strain, and, to a limited degree, strains of HAdV-E4 and B7 (Purkayastha et al., 2005c) contain relatively stable genomes that are remarkably similar at the nucleotide level across time and space, accumulating relatively small genome changes through indels and base substitutions. These do not diminish the HAdV-B3 pathogenic properties apparently, as the pathogen maintains its presence and virulence in the population across a minimum of 50 years.

Why do these HAdV-B3 genomes show so little variability over five decades? One reason is the proofreading capability of the adenovirus DNA polymerase, which enhances the replication fidelity of DNA genome replication (King et al., 1997). Other constraints remain to be defined and understood, particularly in the context of the recombination events that are possible and have been shown for other HAdV genomes.

Despite the high similarity between the field strain and the prototype genomes, there are interesting differences. One is a 20.6 -kDa protein found in the prototype strain, but is truncated after 18 amino acids in the field strains. Another is the E2B 19 kDa protein found in the prototype strain, but is truncated after 5 amino acids in the field strains. A third protein is an E3 9 kDa protein that is found in the prototype strain, but is truncated in the field strains. These truncations occur in the same place within the genes and may be indicative of mutation hotspots. In the NHRC 1276 field strain, there is a 12.6 kDa protein which is truncated after 89 amino acids in the prototype strains and after 81 amino acids in the GZ1 and GZ2 field strains. The truncation occurs in the same place in the GZ1 and GZ2 strains; however, this is not surprising given how similar the two are and given that they were isolated from the same outbreak. Further wet bench analysis needs to be performed on these proteins to elucidate their functions and importance.

With the increasing applications of the genomics and bioinformatics approach to HAdV genomes, coupled with the presence of more cost-effective rapid DNA sequencing technologies, detailed insights into the molecular recombination and/or the overall stability of genomes and these roles in driving the evolution of HAdV types and their pathogenicity and pathoepidemiology are forthcoming, with much greater resolution. This will lead to an understanding of the emergence of novel HAdV pathogens, allowing preparations to be made in identifying and performing surveillance for these pathogens, and to counter them, perhaps controlling epidemic outbreaks with effective and appropriate vaccines.

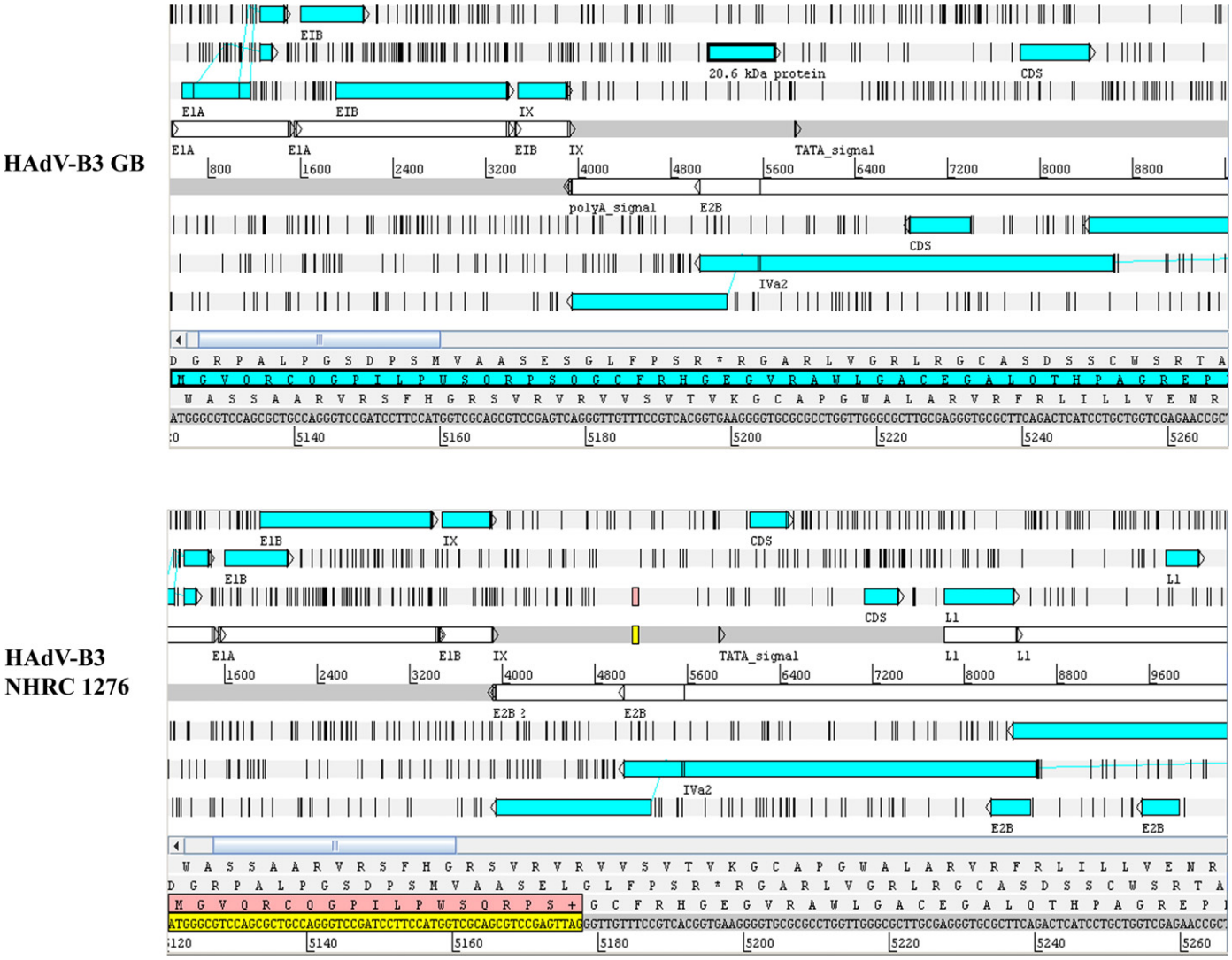


Fig. 4. *In silico* proteome analysis. A genome annotation tool, Artemis, is used to visualize, display and compare the annotation of two genomes. Landmarks such as coding sequences are displayed. HAdV-B3p differs from HAdV-B3a by the 20.6 kDa protein, noted in the top panel, which is truncated by a SNP, e.g., nonsense mutation (noted in the amino acid and nucleotide sequences of the bottom panel), in all three field strains. Both reference genomes of HAdV-B3p have the full-length protein (noted in the amino acid and nucleotide sequences in the top panel). The SNP was confirmed by resequencing the region in both genomes.

Materials and methods

Virus growth, in A549 cells, and DNA production were outsourced to Virapur, LLC (San Diego, CA). Genome sequencing was provided by Commonwealth Biotechnologies, Inc. (Richmond, VA), using similar protocols and strategies for a series of HAdV genomes (Lauer et al., 2004; Purkayastha et al., 2005a,b,c). In brief, Sanger-based chemistry using DYEnamic ET Terminator Cycle Sequencing (Amersham Biosciences; Piscataway, NJ) was resolved on an ABI Prism 377 Sequencer (Applied Biosystems; Foster City, CA), providing average coverage of 5×, minimum 3×, with both strands sequenced. Unreliable data were re-sequenced for resolution, either moving the primers and/or repeating the complementary strand. Additional sequencing QC was provided by genome annotation, resulting in PCR-based, primer-driven resequencing to clarify any ambiguities. Bioinformatics are performed as noted earlier, using similar protocols (Lauer et al., 2004; Purkayastha et al., 2005a,b,c).

HAdV-B3p GB strain was obtained from ATCC (#VR-3; Manassas, VA) (GenBank AY599834); field strain NHRC 1276 (November 1997), was obtained from Naval Health Research Center (Kevin Russell; San Diego, CA) (GenBank AY599836). Another GB genome is available from GenBank, noted as a laboratory strain from T. Adrian (Germany) (GenBank DQ086466) (Sirena et al., 2005); the extent of laboratory passages was not available as the strain was obtained from another researcher who is no longer active in the field (personal communications). This strain is referred to in this work as HAdV-B3 “GB2”. Two field strains, GZ1 and GZ-2, isolated from an outbreak in China (GenBank DQ099432 and DQ105654) are available and documented in the literature (Zhang et al., 2006).

PipMaker (http://pipmaker.bx.psu.edu/cgi-bin/pipmaker?basic) was used to compute alignments of whole genome sequences and to produce dot plots (Schwartz et al., 2000). Local alignments of the genome sequences using BlastZ (Schwartz et al., 2003) and plots of the gap-free segments of the alignments as diagonal lines on the dot plot are generated. Whole genome percent nucleotide identities and single protein amino acid percent identities were calculated using the EMBOSS package (Rice et al., 2000). The genome viewer Artemis was used to view, annotate and compare adenovirus genomes with each other (Berriman and Rutherford, 2003).

The whole genome alignments were performed using MAVID (http://baboon.math.berkeley.edu/mavid) (Bray and Pachter, 2004). A whole genome phylogenetic tree was constructed using MEGA4 and a neighbor-joining algorithm (Tamura et al., 2007; Saitou and Nei, 1987) using the whole genome alignment obtained from MAVID.

1000 bootstrap replications were used to assess the robustness of the tree. TSAdV-1 (tree shrew adenovirus) was used as the "out group" sequence since it is a non-primate mammal.

Genome recombination analysis was performed using SimPlot (Lole et al., 1999). The default parameters used for the BootScan algorithm implemented in Simplot are: window size, 200 bp; Gap-Strip, on; step size, 20 bp; number of repetitions, 100; tree method, neighbor; and distance model, Kimura 2-parameter. To compare these particular genomes with other whole genome data in the literature, the pDRAW32 software (http://www.acaclone.com/) was used for *in silico* RE analysis.

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